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3

Quantitation of 4-Hydroxynonenal Protein Adducts

Koji Uchida and Earl R. Stadtman*

1. Introduction

Lipid peroxidation has been associated with important pathophysiological events in a variety of diseases, drug toxicities, and traumatic or ischemic injuries. It has been postulated that free radicals and aldehydes generated during this process may be responsible for these effects because of their ability to damage cellular membrane, protein, and DNA. Recent studies have shown that the cytotoxicity of products of lipid peroxidation is due in part to the formation of α,β-unsaturated aldehydes, especially 4-hydroxy-2-alkenals *(1,2)*. 4-Hydroxy-2-alkenals elicit a variety of cytopathological effects, including inactivation of enzymes *(1)*, lysis of erythrocytes *(2)*, chemotactic activity toward neutrophils *(3)*, and inhibition of protein and DNA synthesis *(4)*. Among the aldehydes formed, 4-hydroxynonenal is the major product of lipid peroxidation and has been suggested to play a major role in liver toxicity associated with lipid peroxidation *(2,5–7)*.

It is generally accepted that 4-hydroxy-2-alkenals exert these effects because of their facile reactivity toward molecules with sulfhydryl groups *(8–10)*. The α,β double bond of 4-hydroxy-2- alkenals reacts with sulfhydryl groups to form thioether adducts via a Michael-type addition. Whereas it was generally accepted that the aldehyde moiety of 4-hydroxynonenal and other 2-alkenals reacts with primary amino groups to form α,β-unsaturated aldimines *(11,12)*. It is now evident that the ε-amino group of lysine residues in proteins may also undergo an Michael addition reactions with the α,β double bond of 4-hydroxynonenal to form secondary amines possessing an aldehyde group *(13)*. Furthermore, the imidazole groups of histidine residues in proteins also undergo Michael addition type of reactions *(14)*. Accordingly, it is not surprising that the modification of low-density lipoprotein (LDL) *(15,16)* and several other

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proteins *(17)* by 4-hydroxynonenal is associated with a significant loss of lysine and histidine residues. It is thus important to establish the procedures to detect products derived from covalent attachment of 4-hydroxynonenal to amino acid side chains in order to understand the mechanism of a large number of biological effects induced by 4-hydroxynonenal.

1.1. Quantitation of 4-Hydroxynonenal Protein Thioether Adducts

Taking advantage of the fact that Raney nickel catalyzes the cleavage of thioether bonds *(18–21)*, a procedure has been developed *(22)* by which lipid-derived protein carbonyl derivatives can be distinguished from protein carbonyl derivatives generated by other reactions, including metal-catalyzed oxidation and glycation reactions *(23)*. The strategy used is illustrated in **Fig. 1**. In this procedure, the aldehyde moiety of the 4-hydroxynonenal thioether adduct is reduced to the corresponding [^{3}H]-labeled dihydroxyalkane by treatment with tritriated borohydride (NaB[^{3}H]H$_4$). The [^{3}H]-labeled dihydroxyalkane is then released from the protein by cleavage (desulfurization) of the thioether bond by means of Raney nickel catalysis. The free [^{3}H]-labeled dihydroxyalkane is then separated from the reaction mixture by extraction with an organic solvent and is quantitated by radioactivity measurement. This method can be used to measure the amount of 4-hydroxynonenal that is conjugated to proteins by a thioether linkage only. It cannot be used to measure that fraction of 4-hydroxynonenal that is bound to proteins via both a thioether linkage and also by a Schiff base linkage, as would occur if the aldehyde moiety of the primary Michael addition product (thioether) undergoes a secondary reaction with the free amino group of lysine residues in the same or a different protein subunit. Upon reduction with NaB[^{3}H]H$_4$, the Schiff base would be converted to a stable [^{3}H]-labeled secondary amine. Subsequent treatment of the complex with Raney nickel will lead to cleavage of the thioether linkage, but the derivatized 4-hydroxynonenal would still be tethered to the protein via the secondary amine linkage and would, therefore, not be extractable by organic solvents.

2. Materials

2.1. Reagents and Equipment

1. *N*-Acetylcysteine and glutathione (Sigma, St. Louis, MO).
2. 4-Hydroxynonenal: A stock solution of *trans*-4-hydroxy-2-nonenal is prepared by the acid treatment (1 *mM* HCl) of 4-hydroxynonenal diethylacetal and stored at –20°C. Concentration of 4-hydroxynonenal stock solution is determined from the molar extinction coefficient of 4-hydroxynonenal at 224 nm (ε = 13,750). The purity of the stock solution should be checked by high-performance liquid chromatography (HPLC) prior to the experiment.
3. Acetonitrile: 0–100% (v/v) in 0.05% trifluoroacetic acid.
4. EDTA: 10 m*M* and 100 m*M*.

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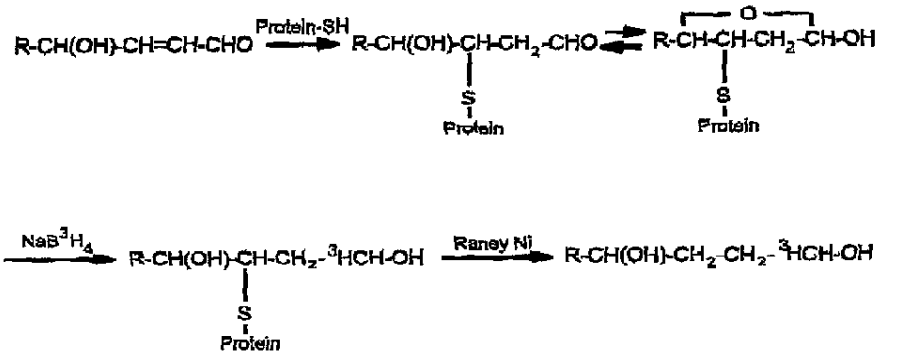

Fig. 1. Experimental scheme to determine 4-hydroxynonenal covalently attached to protein sulfhydryl groups by means of thioether linkage. R:$CH_3(CH_2)_4$⁻.

5. NaOH: 100 m*M* and 1 *M*.
6. HCl: 1 *M* and 6 *M*.
7. Methanol.
8. Sodium phosphate buffer 50 m*M*: pH 7.2 and pH 8.0.
9. Tricholoroacetic acid 20% (w/v).
10. NaB[^{3}H]H_4, 100 m*M*, in NaOH, specific activity about 100 mCi/mmol: Add required volume of 1 *M* $NaBH_4$ and 0.1 *N* NaOH to the NaB[^{3}H]H_4 and store at –20°C.
11. Guanidine HCl, 8 *M*, with 13 m*M* EDTA and 133 m*M* Tris, adjusted to pH 7.2 with HCl.
12. Guanidine hydrochloride 6 *M*.
13. The Raney nickel-activated catalyst (Sigma): Rinse thoroughly with water and ethanol prior to use (*see* **Note 1**).
14. Chloroform/methanol 9:1.
15. Sodium sulfate (dehydrated).
16. *N*-Acetylhistidine and *N*-acetyllysine (Sigma).
17. 0.05% Trifluoroacetic acid (solvent A) as a 100–0% gradient in acetonitrile (solvent B).
18. HPLC: Reverse-phase HPLC is performed on a Hewlett Packard Model 1090 chromatograph equipped with a Hewlett Packard model 1040A diode-array ultraviolet (UV) detector.
19. o-Phthaldialdehyde (OPA, Sigma): Dissolve 3.09 g boric acid in 40 mL HPLC-grade water and adjust the pH to 10.4 with KOH pellets. Dissolve 134 mg OPA in 0.75 mL HPLC-grade methanol in a Sarstedt-type tube and then add to the borate. Add 0.21 mL β-mercaptoethanol, water, and KOH to bring the pH back to 10.4 and the volume to 50 mL. Pipet into a 1.5-mL Sarstedt tube fitted with an O-ring and a cap and store at –20°C.
20. Brij-35: Make 10 mL by mixing 250 mL 2 *M* NaCl, 167 µL 30% Brij-35, and water (*see* **Note 2**).

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3. Methods

3.1. Preparation of [^{3}H]-Labeled 4-Hydroxynonenal Adducts of N-Acetylcysteine and Glutathione

1. To prepare standard samples of 4-hydroxynonenal-cysteine adduct, 2 m*M* *N*-acetylcysteine or glutathione is incubated with 2 m*M* 4-hydroxynonenal in 1 mL of 50 m*M* sodium phosphate buffer (pH 7.2) at 37°C for 2 h. Formation of 4-hydroxynonenal-*N*-acetylcysteine and 4-hydroxynonenal-glutathione adducts can be followed by reverse-phase IIPLC on an Apex Octadecyl 5U column. The adducts are eluted at 1 mL/min with a linear gradient of 0–100% (v/v) acetonitrile in 0.05% trifluoroacetic acid for 25 min. The elution profiles are monitored by the absorbance at 210 nm. Under these chromatographic conditions, the 4-hydroxynonenal-*N*-acetylcysteine and 4-hydroxynonenal-glutathione adducts are eluted at 10.7 and 11.0 min, respectively *(22)*.
2. Take an aliquot (400 µL) of the reaction mixture and add 10 mM EDTA (40 µL)/1 *M* NaOH (40 µL)/100 m*M* NaB[^{3}H]H$_4$ (40 µL) in a 1.5-mL Sarstedt tube fitted with an O-ring and a cap. After incubation for 1 h at 37°C, terminate the reaction by adding 200 µL of 1 *N* HCl, allow to stand 5 min in the hood, and then load the mixture on a Sep-Pak C-18 cartridge. Elute with 5 mL water followed by 5 mL of methanol. Concentrate the methanol fraction in a vacuum centrifuge (Savant), redissolve in 200 µL of methanol, and apply to reverse-phase-HPLC to purify the [^{3}H]-labeled adducts.
3. Collect the eluted fractions in 1-mL aliquots and monitor the amount of [^{3}H] in each fraction by scintillation counting. Collect the fractions which contain the [^{3}H]-labeled products, concentrate, and then redissolve the adducts in 50 m*M* sodium phosphate buffer (pH 7.2). This solution should be stored at –20°C.

3.2. Preparation of [^{3}H]-Labeled 4-Hydroxynonenal-Modified Proteins

1. Incubate 1 mg protein with 2 m*M* 4-hydroxynonenal in 1 mL of 50 m*M* sodium phosphate buffer (pH 7.2) to prepare 4-hydroxynonenal-modified proteins.
2. After incubation for 2 h at 37°C, take an aliquot (400 µL) of 4-hydroxynonenal-modified protein and add an equal volume of 20% trichloroacetic acid (w/v, final concentration).
3. Centrifuge the tubes in a tabletop microcentrifuge at 11,000*g* for 3 min and discard the supernatant.
4. Dissolve the precipitate with 400 µL of 8 *M* guanidine HCl/13 m*M* EDTA/133 m*M* Tris (pH 7.2) and treat with 0.1 *M* EDTA (40 µL)/1 *N* NaOH (40 µL)/0.1 *M* NaB[^{3}H]H$_4$ (40 µL) in a 1.5-mL Sarstedt tube fitted with an O-ring and a cap.
5. After incubation for 1 h at 37°C, add 1 *N* HCl (100 µL) to terminate the reaction and then apply to the PD-10 column (Sephadex G-25), equilibrated in 6 *M* guanidine HCl, in order to separate protein-bound counts from free radioactivity.
6. Collect every 500 µL and determine protein recovery spectrophotometrically and radioactivity by liquid scintillation counting.

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3.3. Raney Nickel Desulfurization

1. Take an aliquot (50 µL) of [^{3}H]-labeled 4-hydroxynonenal-*N*-acetylcysteine adduct, 4-hydroxynonenal-glutathione adduct, or 4-hydroxynonenal-modified proteins prepared as earlier and mix with 400 mg of Raney nickel and 300 µL of 8 *M* guanidine HCl/13 m*M* EDTA/133 m*M* Tris (pH 7.2) in a 1.5-mL Sarstedt tube fitted with an O-ring and a cap.
2. After incubation for 15 h at 55°C, released products are extracted twice with 500 µL each of chloroform/methanol (9:1). Add the chloroform/methanol solution to the mixture, vortex vigorously, and centrifuge the tubes in a tabletop microcentrifuge at 11,000*g* for 3 min.
3. After centrifugation, collect chloroform (lower) layer and add dehydrated sodium sulfate (50–100 mg) to the chloroform solution.
4. After vortexing, take an aliquot (50 µL) of chloroform extract, mix with 5 mL of scintillation liquid, and count the radioactivity to measure the amount of released product (*see* Notes 3 and 4).

3.4. Quantitation of 4-Hydroxynonenal-Histidine and 4-Hydroxynonenal-Lysine Adducts by HPLC

The oxidation of low-density lipoprotein (LDL) is accompanied by a loss of histidine and lysine residues *(24,25)* and its conversion to a form that is taken up by macrophages, giving rise to foam cells that have been implicated in atherogenesis *(25)*. A role of 4-hydroxynonenal in LDL oxidation and its possible involvement in atherogenesis is underscored by the observations: (1) 4-hydroxynonenal is formed during the oxidation of LDL *(12)*; (2) treatment of unoxidized LDL with 4-hydroxynonenal leads to the modification of lysine and histidine residues *(15,16)* and to the conversion of LDL to a form that is taken up by the scavenger receptor on macrophages *(26)*. The development of procedures for the detection and quantitation of the 4-hydroxynonenal-lysine and histidine adducts in proteins is therefore fundamental to assessment of the role such adducts have in the cytotoxicity of lipid peroxidation and atherogenesis.

3.4.1. Sample Preparation

1. *N*-Acetylhistidine and *N*-acetyllysine are used to prepare standard samples of 4-hydroxynonenal-histidine and 4-hydroxynonenal-lysine adduct, respectively. Treat 50 mg of *N*-acetylhistidine (or *N*-acetyllysine) with 5–10 m*M* 4-hydroxynonenal in 2 mL of 50 m*M* sodium phosphate buffer (pH 7.2) for 20 h at 37°C.
2. The products are isolated by reverse-phase HPLC, using a TSK-GEL ODS-80 TM column (0.46 × 25 cm), and a linear gradient of 0.05% trifluoroacetic acid in water (solvent A)–acetonitrile (solvent B) (time = 0, 100% A; 20 min, 0% A), at a flow rate of 1 mL/min. Under these chromatographic conditions, 4-hydroxynonenal adducts of *N*-acetylhistidine and *N*-acetyllysine are eluted at 11.2 min and 10.8 min, respectively.
3. 4-Hydroxynonenal-modified proteins are prepared according to the procedures described earlier.

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3.5. Acid Hydrolysis of 4-Hydroxynonenal-Modified Samples

1. An aliquot (0.1 mL) containing 0.1 mg of 4-hydroxynonenal-modified proteins or 1–10 nmol of purified 4-hydroxynonenal-*N*-acetylhistidine or 4-hydroxynonenal-*N*-acetyllysine adduct is placed in a hydrolysis vial.
2. Add 10 µL each of 10 m*M* EDTA, 1 N NaOH, and 0.1 *M* $NaBH_4$, and incubate for 1 h at 37°C (*see* **Note 5**).
3. Add 30 µL of 1 *N* HCl to terminate the reaction and concentrate in a vacuum centrifuge.
4. Add 200 µL of 6 *N* HCl and flush the headspace with nitrogen for 60 s, and then cap using a screw cap fitted with a 12-mm Teflon/silicone liner with the Teflon facing the HCl.
5. Place the vial in a benchtop heater at 110°C and incubate for 20 h.
6. Concentrate the mixture in a vacuum centrifuge and redissolve in the desired volume of 50 m*M* sodium phosphate buffer (pH 8.0) containing 1 m*M* EDTA.

3.6. Amino Acid Analysis

3.6.1. Derivatization

1. Pipette 10 µL *o*-phthaldialdehyde onto the bottom of a Sarstedt tube.
2. Tilt the tube and place 10 µL of the sample on the side of the tube and mix with a vortex and start the timer.
3. At 0.25 min, add 180 µL Brij-35.
4. At 1 min, inject 100 µL of the above mixture and start the gradient.

3.6.2. HPLC Analysis

Reverse-phase HPLC is performed on a Hewlett Packard Model 1090 chromatograph equipped with a Hewlett Packard model 1046A programmable fluorescence detector (excite at 340 nm and follow emission at 450 nm). The column we used to use is a 15 cm C_{18} from Jones Chromatography (5 µ size). Control the column temperature at 30°C. The flow rate is 2.0 mL/min. The following gradient program is used. All gradients are linear (A: 100 m*M* NaCl, B: water, C: CH_3OH)

Time (min)	Gradient
0.0	A = 50.0%, B = 50.0%
0.3	A = 37.5%, B = 37.5%, C = 25.0%
7.0	A = 37.5%, B = 37.5%, C = 25.0%
11.0	A = 30.0%, B = 30.0%, C = 40.0%
12.0	A = 25.0%, B = 25.0%, C = 50.0%
16.0	A = 22.5%, B = 22.5%, C = 55.0%
18.0	A = 12.5%, B = 12.5%, C = 75.0%
20.0	A = 12.5%, B = 12.5%, C = 75.0%
21.0	A = 50.0%, B = 50.0%

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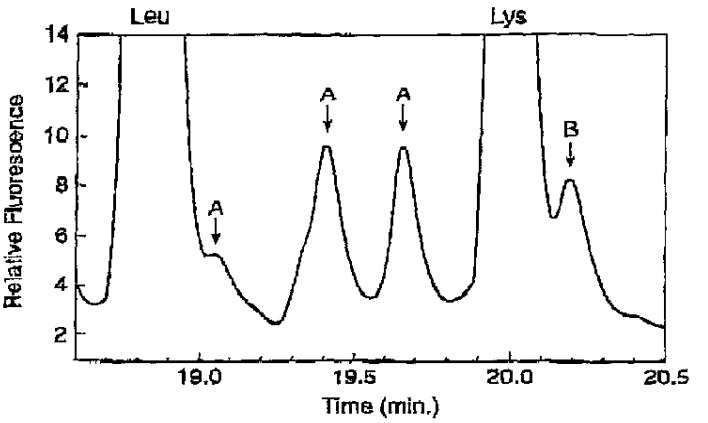

Fig. 2. Separation of the 4-hydroxynonenal adducts by HPLC. The enzyme (1 mg/mL) was incubated with 2 mM 4-hydroxynonenal in 50 m*M* sodium phosphate buffer (pH 7.2) for 2 h at 37°C. After reduction with $NaBH_4$, reaction mixtures were hydrolyzed and the amino acid composition was analyzed by HPLC following derivatization with OPA. The arrows in section A indicate the peaks corresponding to the 4-hydroxynonenal-histidine adducts. The arrow in B indicates the 4-hydroxynonenal-lysine peak. The peaks marked Leu and Lys correspond to the OPA derivatives of leucine and lysine, respectively.

In the HPLC system used, *o*-phthaldialdehyde derivatives of the reduced forms of 4-hydroxynonenal-histidine and 4-hydroxynonenal-lysine adduct can be separated from all other normal amino acids. As shown in Fig. 2, the histidine derivatives appear as three separate peaks (isomers?) which elute between leucine and lysine at 19.05, 19.4, and 19.7 min; whereas the lysine adduct elutes just after lysine at 20.2 min. By integration of the areas under these peaks and comparison with the areas of amino acid standards, the amounts of histidine and lysine 4-hydroxynonenal adducts can be quantitated.

3.7. Concluding Remarks

We found that the major product formed in the reaction of 4-hydroxynonenal with α-*N*-acetyllysine or poly-L-lysine is the Michael addition product; that is, a secondary amine produced by addition of the ε-amino group of lysine to the double bond (C_3) of 4-hydroxynonenal *(13)*. For a number of 4-hydroxynonenal-modified proteins tested (glyceraldehyde phosphate dehydrogenase, insulin, bovine serum albumin, low-density lipoproteins), the number of 4-hydroxynonenal-histidine residues detected by these procedures was almost exactly equal to the number of histidine residues that disappeared.

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However, the number of lysine–hydroxynonenal and cysteine–hydroxynonenal adducts that can be detected by HPLC assay of the *o*-phthaldialdehyde derivatives and the Raney nickel treatment, respectively, is often considerably lower than the number of these residues that disappeared upon 4-hydroxynonenal treatment. For example, reaction of 4-hydroxynonenal with glyceraldehyde-3-phosphate dehydrogenase under the conditions described here led to the modification of 5 histidine, 3.5 lysine, and 2.5 cysteine residues *(17)*. By means of the Raney nickel procedure, only 17% of the modified cysteine could be attributed to a simple Michael addition reaction, whereas 90% and 28%, respectively, of the histidine and lysine residues were present as simple Michael addition products, as determined by HPLC of the *O*-phthaldehyde derivatives of $NaBH_4$-treated acid-hydrolyzed samples. It was proposed that the poor recovery of lysine and cysteine residues might be due to secondary reactions in which the aldehyde groups of some primary Michael addition products react with proximal lysine residues to form Schiff-base crosslinks, which would be stabilized by reduction with $NaBH_4$ *(17)*. This possibility is supported by (1) the observation that the number of cysteine plus histidine residues that could not be accounted for as Michael addition products is equal to the number of lysine residues that could not be accounted for and (2) by the appearance of protein conjugates that upon SDS gel electrophoresis exhibited molecular weights about two times that of the native subunit *(17)*.

4. Notes

1. Avoid complete drying because raney nickel is inflammable.
2. Make this solution up on the day of use, as the Brij-35 is not stable.
3. Upon treatment with Raney nickel, the [^{3}H]-labeled product can be recovered in 80–90% yield from both 4-hydroxynonenal-*N*-acetylcysteine and 4-hydroxynonenal-glutathione adducts in a solvent 10% methanol/chloroform-extractable form *(22)*. Before Raney nickel treatment, only 1% and 25% of the radioactivity in the *N*-acetylcysteine and glutathione adducts, respectively, were extracted by the solvent. It was considered that the small amount (25%) of solvent-extractable radioactivity present in the 4-hydroxynonenal-glutathione derivative before Raney nickel treatment is due to the contamination of the adduct preparation with radiolabeled reduced forms of free 4-hydroxynonenal.
4. When glyceraldehyde-3-phosphate dehydrogenase which contains 4 sulfhydryl groups per subunit, was modified with 4-hydroxynonenal, 3.2 of these sulfhydryl groups are lost; however, 0.54 mol/mol of the labeled adduct was released in a solvent-extractable form upon treatment with Raney nickel. Therefore, only 17% of the modified cysteine residues is present as a simple 4-hydroxynonenal-thioether adduct. Upon HPLC analysis, [^{3}H]-labeled product released after treatment with Raney nickel was indistinguishable from the products obtained following Raney nickel treatment of the 4-hydroxynonenal-*N*-acetylcysteine and

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4-hydroxynonenal-glutathione adducts. Thus, the procedure using [^{3}H]-labeling followed by Raney nickel treatment enables one to determine bonafide 4-hydroxynonenal-protein thioether adducts and attests to the fact that the reaction of 4-hydroxynonenal with cysteine residues of proteins may lead to derivatives other than simple thioether adducts (*see* **Subheading 3.7.**).

5. Treatment of the 4-hydroxynonenal-histidine and 4-hydroxynonenal-lysine adducts with $NaBH_4$ stabilizes the histidyl–4-hydroxynonenal linkage. If the reduction step is omitted, acid hydrolysis of the 4-hydroxynonenal adducts leads to quantitative release of the histidyl and lysyl moieties as free amino acids. These observations are consistent with the Michael addition mechanism, provided that reversal of the addition reaction is catalyzed by strong acid. Thus, the reduction of the aldehyde moiety would preclude reversibility and, thereby, lead to formation of an acid-stable derivatives.

Acknowledgment

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4

Detection Using Dic

Cecile M. Kr

1. Introducti

1.1. General

Normal oxi species (ROS) uct hydrogen chain *(1)*. In a specialized en genase, and lip oxides [ROOH of mechanism lead to increa cycling of met cal (RS·) prod cants may dire capacity by de chrome P450 (ways, and thr cells with dim of ROS may c oxidative stres

Many meth described, incl assays of othe by ROS, such aldehydes, pro

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